Synergistic Effects of Cyclic AMP and Nerve Growth Factor on Neurite Outgrowth and Microtubule Stability of PC12 Cells

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ABSTRACT The outgrowth of neurites from rat PC12 cells stimulated by combined treatment of nerve growth factor (NGF) with cAMP is significantly more rapid and extensive than the outgrowth induced by either factor alone. We have compared the responses of PC12 cells under three different growth conditions, NGF alone, cAMP alone, and combined treatment, with respect to surface morphology, rapidity of neurite outgrowth, and stability of neurite microtubules, to understand the synergistic action of NGF and cAMP on PC12. Surface events at early times in these growth conditions varied, suggesting divergent pathways of action of NGF and cAMP. This suggestion is strongly supported by the finding that cells exposed to saturating levels of dibutyryl cAMP without substantial neurite outgrowth initiated neurites within 5 min of NGF. This response has been adopted as a convenient assay for NGF. Neurites that regenerated in the three growth conditions showed marked differences in stability to treatments that depolymerize microtubules. The results indicate that microtubules in cells treated with both NGF and cAMP are significantly more stable than in either growth factor alone. We suggest that a shift of the assembly equilibrium favoring tubulin assembly is a necessary prerequisite for the initiation of neurites by PC12.

The cytoskeleton is known to play a crucial role in the specification and maintenance of cell shape. The factors that organize the cytoskeleton and integrate the various filamentous components are poorly understood. The neuron is an unusually interesting system for the study of cytoskeleton function underlying cellular morphology. The exaggerated processes, axons and dendrites, of neurons are critically important for the function of the cells. The outgrowth of the axon is dependent upon the actin-based motility of the growth cone (24, 37, 38) and can be altered by a number of factors in the environment, including substrate adhesion (25) and nerve growth factor (NGF)[1] gradients (8). In addition, the advance of the growth cone requires the presence of the axonal microtubules (MTs) (11, 24, 37). The mechanism by which growth cone motility is appropriately integrated with axonal MT assembly is unknown. Indeed, the spatial organization of axonal MTs is intriguing in other ways. In most cells, MT organization depends on a microtubule organizing center (27). Although axonal MTs are discontinuous (4, 36) and of uniform polarity orientation (7, 20), no clear candidate exists for an axonal-microtubule organizing center.

PC12 cells are a clonal cell line derived from a rat pheochromocytoma of the adrenal medulla (14). PC12 can be induced to differentiate into cells with typical neuronal morphology by a number of substances, including NGF (14, 26) and cAMP (33, 34). Addition of both substances to PC12 produces a synergistic response; neurite outgrowth is more rapid and is transcription- and translation-independent (16). Neurite outgrowth independent of macromolecular synthesis indicates that only reorganization of existing cytoplasmic material is required and suggests that this synergistic response is likely to be a favorable system to investigate the cytoskeletal mechanisms underlying neurite outgrowth. A study of this

[1] *Abbreviations used in this paper*: dbcAMP, N^6, O^2-dibutyryl 3′, 5′-cyclic adenosine monophosphate; MT, microtubule; N-cultures, containing NGF; NC-cultures, containing NGF and chloroadenosine or dbcAMP; NGF, nerve growth factor.

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synergistic response may also illuminate the current controversy concerning the role of cAMP in NGF-stimulated responses (16, 18, 33–35).

We report here an investigation of the synergistic effects of NGF and cAMP on the morphology of the membrane and the stability of MTs of PC12 cells using phase microscopy, scanning electron microscopy, and transmission electron microscopy.

MATERIALS AND METHODS

Materials: NGF was prepared from mouse saliva as previously described (12). N^6, O^2-dibutyryl 3′,5′-cyclic adenosine monophosphate (dbcAMP), 2-chloroadenosine, and cholera toxin were purchased from Sigma Chemical Co. (St. Louis, MO). Nocodazole was obtained from Aldrich Chemical Co. (Milwaukee, WI). Calcium, magnesium-free phosphate buffered Gey's solution was prepared as described (21).

Culture of PC12 Cells: PC12 cells originally isolated by Greene and Tischler (14) were obtained from the laboratory of Dr. Eric Shooter, Stanford University. Cells were maintained on polystyrene tissue culture dishes (Corning Medical and Scientific, Medfield, MA) or on Primaria tissue culture dishes (Falcon Labware, Oxnard, CA) coated with polylysine in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 10% horse serum and 5% newborn calf serum (North American Biologicals, Inc., Miami, Fla.) at 37°C in a humidified atmosphere containing 10% CO_2. Cells were passed at a 1:4 split ratio. Addition of NGF (50 ng/ml), dbcAMP (1 mM), or 2-chloroadenosine (0.1 μM) to cells was done using stocks of 100 times the final concentration in calcium, magnesium-free phosphate buffered Gey's solution.

Scanning Electron Microscopy: Cells were grown on plastic tissue culture coverslips (Lux Scientific, Inc., Newbury Park, CA) previously coated with protamine by soaking 30 min in 1 mg/ml protamine sulfate (Sigma Chemical Co.) in water followed by extensive washing in water. After experimental treatment, medium was removed from cells, and cells were immediately fixed with 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4. Cells were fixed for 1 h, washed twice in 0.15 M sodium cacodylate, pH 7.4, postfixed 90 min in OsO_4 (1% in 0.15 M cacodylate buffer), washed 18 h in 0.15 M cacodylate, dehydrated with successively increasing concentrations of ethanol, and critical-point dried and sputter-coated with a 300-Å layer of gold-palladium. Cells were observed with a JEOL JSM 30 scanning electron microscope at a 0° tilt angle.

Transmission Electron Microscopy: Cells were grown on 60-mm tissue culture dishes (Falcon Labware) to 1×10^5 cells/plate. After experimental treatment with dbcAMP, NGF, etc., the monolayers were washed twice with phosphate-buffered saline and fixed with 3% glutaraldehyde in phosphate-

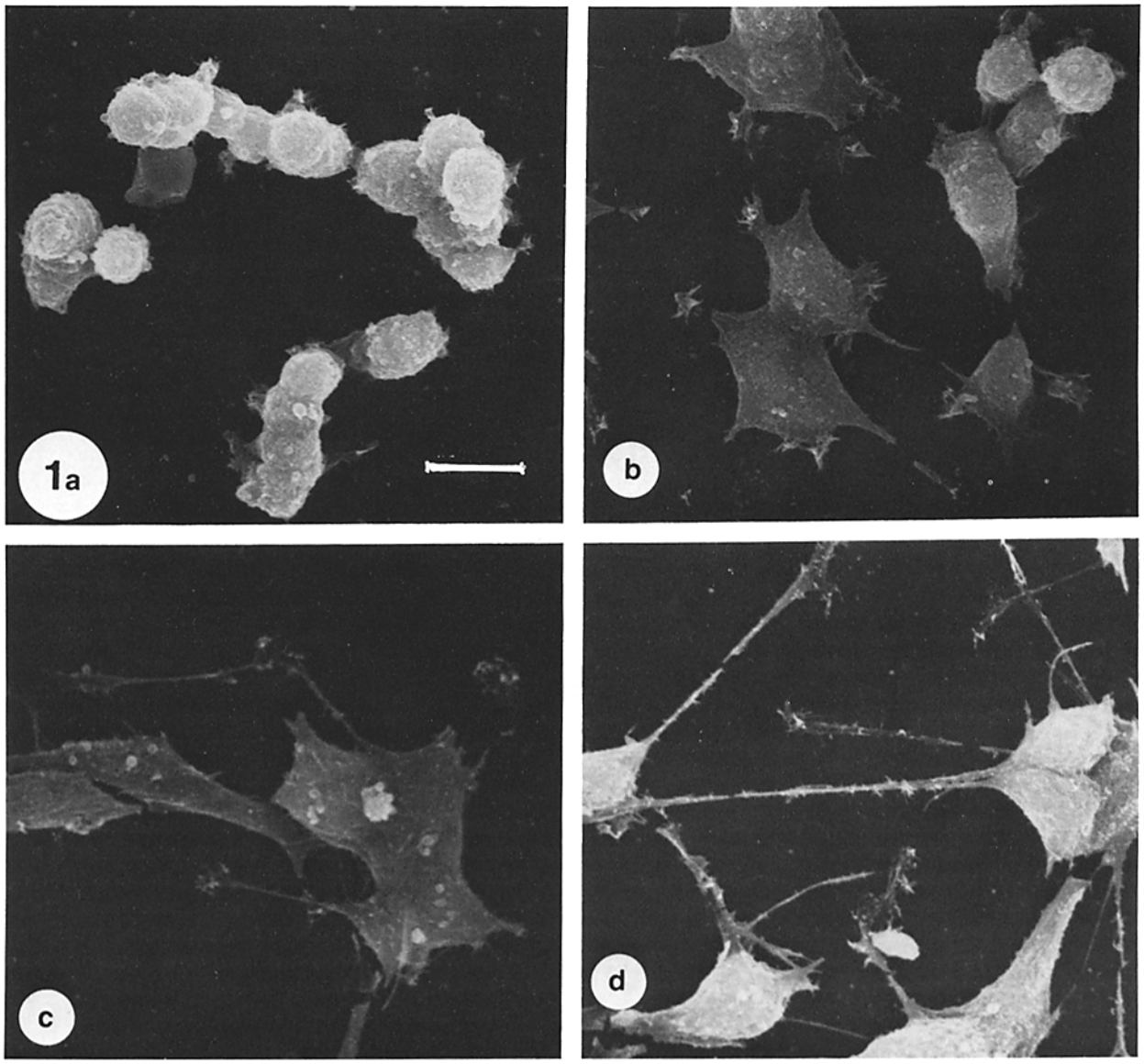

FIGURE 1 Effect of dbcAMP on PC12 cell morphology. PC12 cells were plated onto plastic tissue culture coverslips and 24 h later were cultured in growth medium for 48 h (*a*) with no additions; (*b*) with 50 ng/ml NGF; (*c*) with 1 mM dbcAMP; (*d*) with 50 ng/ml NGF and 1 mM dbcAMP. Cells were fixed, prepared, and observed in the scanning electron microscope as described in Materials and Methods. × 1,400. Bar, 10 μm.

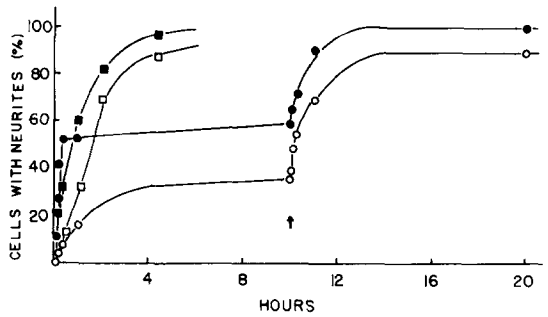

FIGURE 2 Time course of neurite production in response to dbcAMP and NGF. Cells were plated on plastic tissue culture coverslips 24 h before the experiment. After addition of dbcAMP, or dbcAMP and NGF, cells were cultured for the time given on the abscissa, then fixed, prepared, and observed in the scanning electron microscope as described in Materials and Methods. Each data point represents, for 200 cells observed, the fraction of cells bearing neurites longer than 0.5 cell diameter (~10 μm) denoted by filled symbols (●, ■) or 1 cell diameter (20 μm) denoted by open symbols (○, □). Processes were identified as neurites by the criteria described in Results. Cells were cultured with 50 ng/ml NGF and 1 mM dbcAMP (□, ■) or were cultured initially in 1 mM dbcAMP with addition of 50 ng/ml NGF at 10 h, denoted by arrow (○, ●). As reported by others (6, 14, 16, 26), we found that process formation requires several days in cultures containing only NGF. Because of this difference in response time, cells treated only with NGF were not included in this study.

buffered saline. After 90 min of fixation, the monolayers were washed and postfixed in 1% OsO_4 in cadodylate buffer. The samples were then dehydrated with successively increasing concentrations of ethanol and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate, and observed using a Phillips 300 transmission electron microscope.

Microtubule Stability: Cells were grown in the presence of NGF for 6 d to 2 wk in 100-mm dishes. After this period of "priming" (6), cells were triturated from the surface and replated (3×10^4 cells/plate) onto 35-mm Primaria tissue culture dishes (Falcon Labware) that had previously been treated with 1 mg/ml polylysine for 30 min and then were rinsed twice with water. Cells were allowed to regenerate neurites in one of three culture conditions, with NGF alone, NGF and elevated cAMP levels (dbcAMP as above or 0.1 μM 2-chloroadenosine), or with dbcAMP only. Before experimental treatments to depolymerize neurite MTs, the cultures were examined with an inverted phase microscope and two areas on each dish were marked with 1.5-mm circles on the bottom of the dish with a diamond object marker objective. Four photographs were taken of different areas within each circled region. These four micrographs surveyed ~65% of the total circled area. Cultures were treated with nocodazole (0.05 μg/ml, 0.1 μg/ml) or with low temperature (4% or 10°C) to depolymerize MTs. Because the low-temperature incubators lacked a 10% CO_2 atmosphere, cultures destined for low-temperature treatment were supplemented with 10 mM HEPES at pH 7.4. At various times after the start of treatment, four photographs were again taken of each circled region. Quantitative data, as in Table I, were obtained by counting the neurites in each micrograph. Unless otherwise noted, the figures reported are the sum of neurites in all eight micrographs from a single dish before or after treatment. At the end of treatment, 10% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, was added to the culture to a final concentration of 2%. After fixation, cells were postfixed and processed for transmission electron microscopy, as described above.

RESULTS

Rapidity and Extent of Process Formation

PC12 cells, cultured on protamine-coated plastic coverslips, appear in the scanning electron microscope as rounded cells densely covered with microvilli (Figs. 1*a* and 3*a*). Cultures maintained for 48 h with either NGF or dbcAMP cause significant flattening of the cells, as well as formation of short processes that are rarely >10 μm long (Fig. 1, *b* and *c*). Cultures maintained for 48 h in the simultaneous presence of NGF and cAMP cause cells to form processes at a greater frequency (approaching 100%) and of a greater length, generally extending to the nearest neighboring cells over distances ≥50 μm (Fig. 1*d*).

Scanning electron microscopy of cells fixed at various times after addition of dbcAMP, or dbcAMP and NGF, was used to estimate the rapidity of the response. At each time point, we observed 200 cells and determined the fraction of cells with processes as long as and half as long as a cell's diameter. Fig. 2 reveals that responses to the addition of dbcAMP or of dbcAMP plus NGF are indeed quite rapid. A significant increase in frequency of occurrence of neurites was seen within 2 min of addition of dbcAMP, and after 20 min, 50% of the cells had neurites. The fraction of cells bearing processes did not increase above that level over the subsequent 10 h. The mean length of neurites increased slightly over this time period, but neurites remained short, rarely >20 μm long. The addition of NGF alone caused no significant initiation of neurites over a 10-h period. However, the addition of NGF to cells previously cultured 10 h in the presence of dbcAMP caused a significant increase in the frequency of occurrence of neurites above that obtained with dbcAMP alone. A significant effect was seen within 5 min, and >90% of cells had neurites by 1 h. By 10 h, virtually every cell had a neurite, and most were >20 μm long. Simultaneous addition of NGF and dbcAMP caused >90% of the cells to initiate neurites within 4 h.

The short processes formed in response to dbcAMP and/or NGF at the earliest stages of response cannot be unequivocally identified as "neurites." They are neuritic in being relatively narrow in cross-section and having active growth cones, but they are not clearly distinguishable from other types of cell processes such as the leading lamellae of migratory fibroblasts. However, sequential light micrographs taken over a period of 24 h revealed that the short processes formed in response to dbcAMP or in early stages of the response to NGF elongate in the simultaneous presence of dbcAMP and NGF to form processes that are clearly neurites. Thus, for the purposes of this discussion, we have identified as "neurites" all processes that are relatively narrow and possess a broadened terminal aspect.

Surface Morphology Changes Accompanying Stimulation by NGF and cAMP

Connolly et al. (9) have used scanning electron microscopy to demonstrate that whereas initiation of neurites by NGF-stimulated PC12 cells require several days, NGF nonetheless does cause a series of changes in cell-surface morphology at much earlier times. These changes include rapid loss of microvilli, transient membrane ruffling that appears centrally at 2 min and moves to the cell periphery by 7 min, and subsequent appearance of large blebs. We have verified these observations. We thought it would be worthwhile to determine what surface morphology changes accompany NGF action under conditions, with added dbcAMP, that cause very rapid initiation of neurite production.

The effects of dbcAMP alone are shown pictorially in Fig. 3 and quantitatively in Fig. 5*a*. dbcAMP caused nearly complete loss of microvilli at the earliest times that could be examined. As seen in Fig. 3*b*, very few microvilli remained when cells were fixed 1 min after dbcAMP addition. A tran-

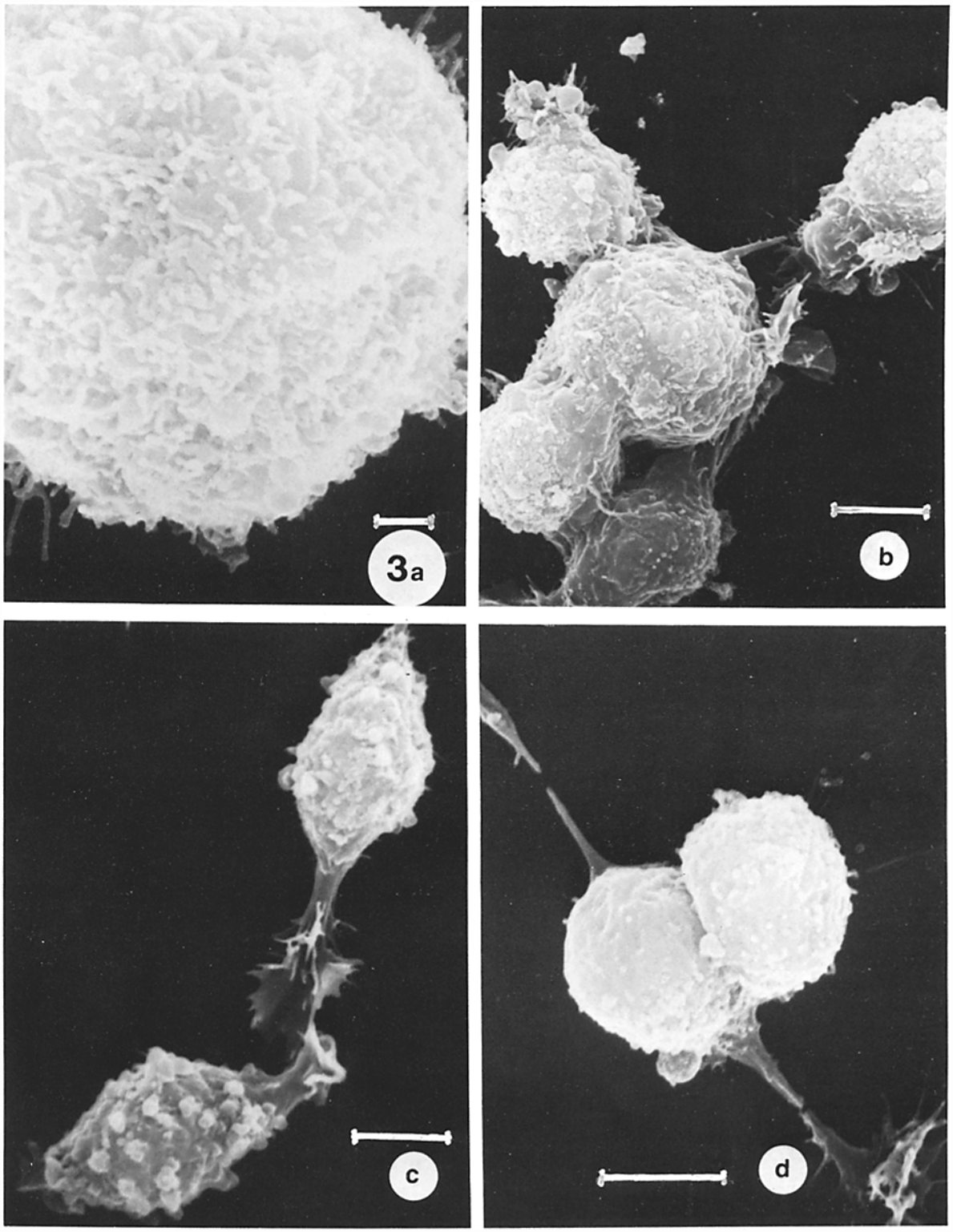

FIGURE 3 Effect of dbcAMP on surface morphology of PC12 cells. Cells plated on plastic coverslips 24 h before the experiment were cultured (*a*) with no additions; or (*b*) with added 1 mM dbcAMP for 1 min; (*c*) for 2 min; or (*d*) for 60 min. Scanning electron photomicrographs were prepared as described in Materials and Methods. (*a*) × 9,400; bar, 1 μm. (*b* and *c*) × 3,200; bar, 5 μm. (*d*) × 4,000; bar, 5 μm.

sient increase in membrane ruffling occurred at 2 min (Figs. 3*c* and 5*a*). At times of 60 min or longer after dbcAMP addition, cells had smooth rippled surfaces with few blebs or microvilli (Figs. 3*d* and 4*a*).

Addition of NGF to cells previously incubated 10 h with dbcAMP caused a series of alterations analogous to those seen upon addition of NGF to untreated cells. Since cells with dbcAMP were initially devoid of microvilli, the NGF-induced loss of microvilli could not be observed (Fig. 4*a*). However, membrane ruffling occurred, first centrally, and then peripherally (Fig. 4*b*) and followed a time course (Fig. 5*b*) that was similar to that seen upon addition of NGF to cells in the

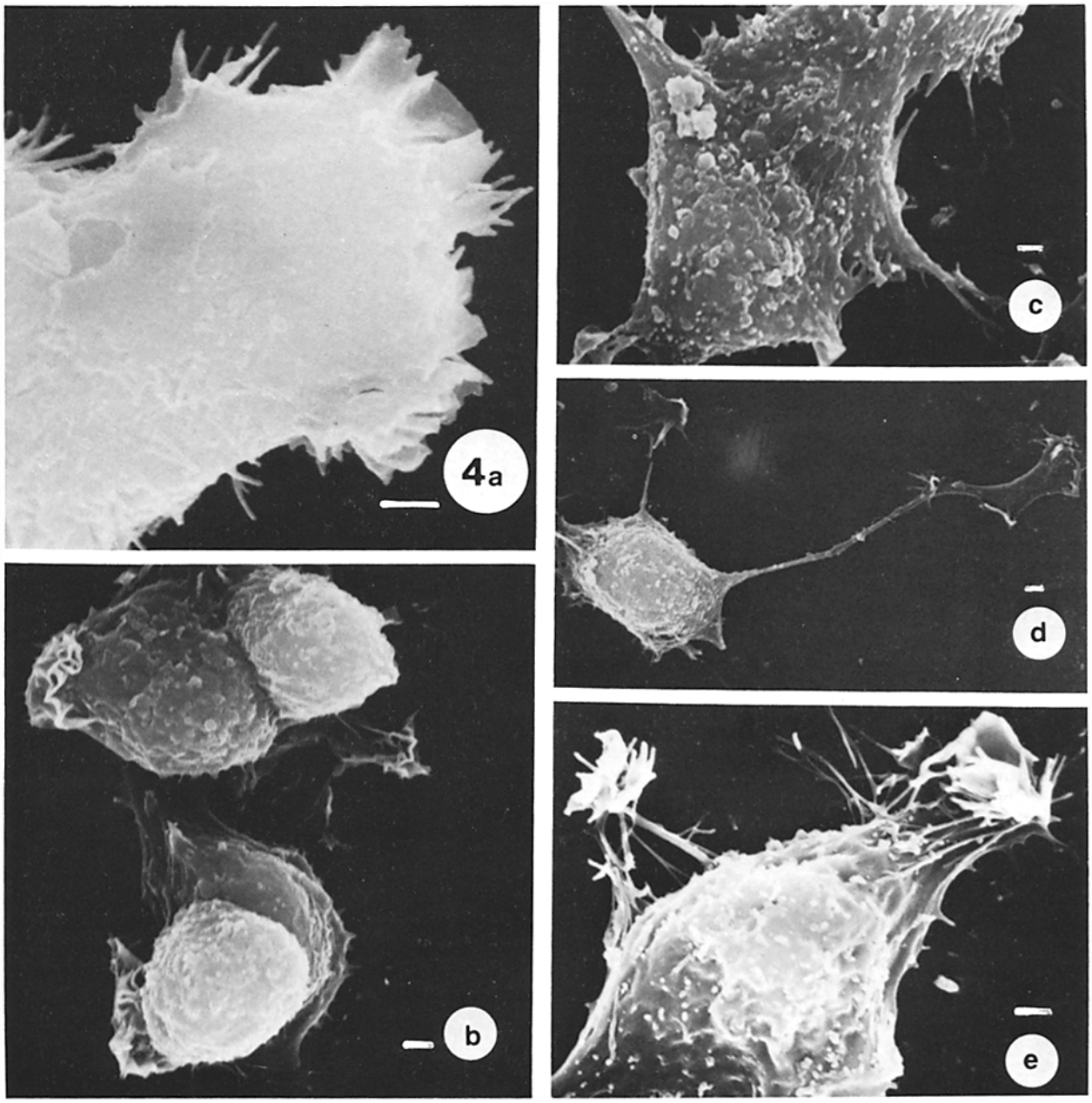

FIGURE 4 Effect of NGF on surface morphology of dbcAMP-treated PC12 cells. PC12 cells on surface coverslips cultured for 24 h with 1 mM dbcAMP and (*a*) no additions; (*b*) added 50 ng/ml NGF for 2 min; (*c*) NGF for 7 min; or (*d*) NGF for 60 min. Cells in *e* were fixed 10 min after simultaneous addition of 1 mM dbcAMP and 50 ng/ml NGF. Scanning electron micrographs were prepared as described in Materials and Methods. Bar, 1 μm. Magnifications: (*a*) 8,300; (*b*) 3,900; (*c*) 3,500; (*d*) 2,600; (*e*) 5,000.

absence of dbcAMP. By 60 min, all ruffling activity ceased and most cells had neurites (Fig. 4*d*).

When dbcAMP and NGF were added simultaneously, a new phenomenon was observed. Transient ruffling still occurred, but it was predominantly localized at the growth cones of neurites (Fig. 4*e*). Observation of a large number of such cells conveyed the impression that ruffling areas at the periphery of cells become sites of initiation of neurites or, alternatively, initiation of neurites suppressed ruffling elsewhere.

Observations with transmission electron microscopy revealed that PC12 processes formed in response to NGF are typically axonal, having abundant MTs, secretion granules, and few ribosomes (26). We used transmission electron microscopy to investigate whether the processes formed in the more rapid response to dbcAMP with NGF are equally axonal in character. As shown in Fig. 6, processes formed in response to cAMP with NGF are quite similar to processes formed in response to NGF alone—they are typically axonal in their content of MTs and in the infrequent occurrence of ribosomes. However, cells treated with NGF and cAMP often contained extensive regions of the neurite that were devoid of secretion (chromaffin) granules as shown in Fig. 6*a*. Virtually every section through neurites stimulated by NGF alone contained several such granules (Fig. 6*b*). Like Luckinbill-Edds et al. (26), we did not observe intermediate filaments in processes of PC12.

Cholera toxin (28) and adenosine analogues (17) are known to elevate intracellular cAMP levels. We found that cholera

toxin and 2-chloroadenosine enhanced the response to NGF in a manner similar to that observed for dbcAMP. NGF rapidly causes neurite formation, in a dose-dependent fashion, from cholera toxin-treated PC12 cells. We have employed this response as a convenient and highly reproducible bioassay for NGF. Fig. 7 shows a dose-response curve for such a bioassay.

Stability of Neurite MTs in Different Growth Conditions

Studies have attributed morphological effects of cAMP (30, 31) and NGF (2) to changes in the organization of MTs. Depolymerization of neurite MTs by colchicine has been shown to cause neurites of the treated cell to retract into the cell body (11, 37). We compared the stability of neurites formed in response to cAMP alone, NGF alone, and NGF with cAMP, to retraction as a result of treatments that depolymerize MTs.

In one experimental design, cells were grown for 6 d with NGF only, then transferred into smaller dishes containing either NGF alone, or NGF and cAMP (containing either 1 mM dbcAMP or 0.1 μM 2-chloroadenosine) and allowed to incubate, i.e., regenerate neurites, for 24 h. To minimize sampling bias in the course of the experiment, small regions of each dish were marked before MT depolymerization. Only these regions were surveyed by photography (see Materials and Methods) before and after experimental treatment. The result of a typical experiment is presented in Table I. Entirely similar results were obtained in two additional experiments. Figs. 8 and 9 show cells before treatment. The addition of 0.05 or 0.1 μg/ml of nocodazole to cultures containing only NGF (hereafter, N-cultures) caused the retraction of >90% of the neurites within 15 min (Fig. 10). In six experiments of scoring N-culture neurites after 15 min in 0.1 μg/ml nocodazole, 67/1,134 neurites remained. In contrast, as shown in Table I, ~85% of the neurites remained in cultures containing NGF and dbcAMP or chloroadenosine (NC-cultures) treated

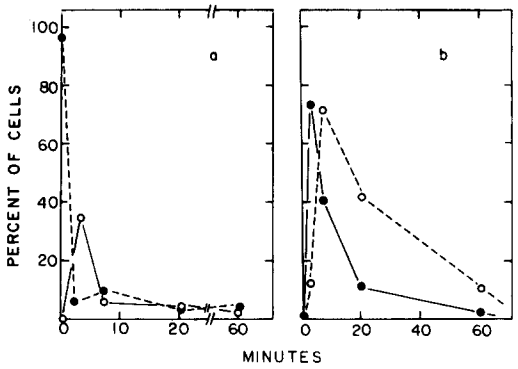

FIGURE 5 Time course of surface morphology responses to dbcAMP or dbcAMP with NGF. PC12 cells were cultured on plastic coverslips for 24 h before the experiment. In *a*, cells were treated for various times with 1 mM dbcAMP only before fixation. In *b*, cells were treated with 1 mM dbcAMP and 50 ng/ml NGF for various times before fixation. Cells prepared as described in Materials and Methods were observed in the scanning electron microscope and each data point represents, for >100 cells, the percentage of cells bearing particular structural features: (*a*) microvilli (●) and ruffles (○); (*b*) central ruffles (●) and peripheral ruffles (○).

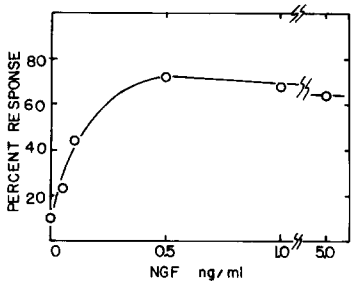

FIGURE 7 NGF bioassay using cholera toxin-treated PC12 cells. PC12 cells were plated at 100,000 cells per well in Costar 24-well plates. After 18 h of culture 10^{-10} M cholera toxin and indicated concentrations of NGF were added, and cells were observed by phase-contrast microscopy 24 h later. Percentage of cells bearing neurites greater than one cell diameter in length is plotted as a function of NGF concentration.

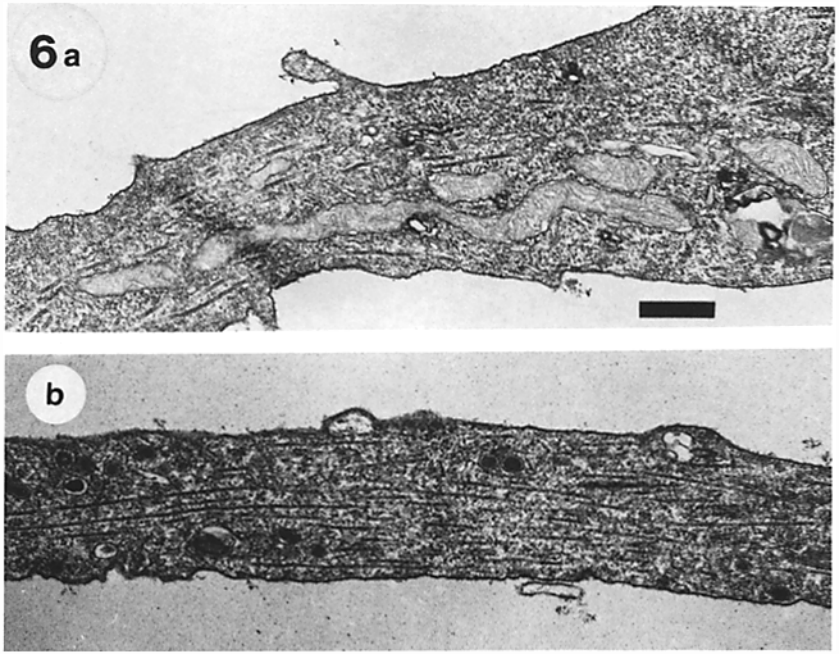

FIGURE 6 Neurites of PC12 cells cultured with NGF or cyclic AMP and NGF. (*a*) Neurite of a PC12 cell cultured for 48 h with 50 ng/ml NGF and 1 mM dbcAMP. A considerable length of many neurites was devoid of chromaffin granules as seen for this region. However, almost all such neurites contained chromaffin granules near the cell body. (*b*) Neurite of a PC12 cell cultured for 7 d with 50 ng/ml NGF. Chromaffin granules were found distributed throughout such neurites. Bar, 0.7 μm. × 14,300.

with 0.1 μg/ml of nocodazole after 15 min (Fig. 11 *a*). Indeed, combined data from four experiments scoring NC-culture neurites after 15 min in 0.1 μg/ml nocodazole showed an even higher fraction of neurites remaining, 1,126/1,179. These neurites were quite stable; 65% remained even after 45 min at 0.1 μg/ml nocodazole (Fig. 11 *b*). Electron microscopic observation of neurites from NC-cultures indicated that MTs persisted in large number as expected from neurite behavior (Fig. 12). NC-culture neurites were also found to be much more stable than N-culture neurites in experiments using low temperature as the MT-depolymerizing treatment. After a 2-h incubation at 4°C, 67% of NC-culture neurites had not retracted (Fig. 13) and electron microscopy indicated the presence of a substantial number of MTs (not shown). However, >90% of N-culture neurites retracted at 4°C and ~85% at 10°C. Fig. 14 shows the effect of 10°C for 2 h on N-cultures.

The N- and NC-cultures used in the experiments above differed only by a 24-h period of "augmentation" by cAMP. Both had equal times of exposure to NGF, 7 d, and an equal period for regeneration of neurites. The more rapid regeneration of neurites in cells with elevated levels of cAMP resulted in neurites of NC-cultures being longer and more numerous than the regenerated neurites of N-cultures at the time of experimental treatment. Preliminary observations suggested to us that neurite retraction was affected by neurite length; the longer the neurite, the more resistant to retraction. An altered experimental design attempted to control for this factor. In this design, all cells had equal time of exposure to NGF, 17 d, but differed in two ways. NC-cultures again had a 24-h period of elevated cAMP levels and N-cultures were allowed to regenerate neurites for 3 d vs. 1 d for NC-cultures. Fig. 15 shows the typical appearance of such an N-culture before experimental treatment. Although the appearance of these cells was not identical to those in NC-cultures (Fig. 9), the neurites were clearly longer and more numerous than after 24 h of regeneration (Fig. 8). These neurites nevertheless

TABLE I

Retraction of Neurites Regenerated for 24 h in Response to Microtubule Depolymerization

Treatment	NGF only*	NGF and cAMP*
0.05 μg/ml nocodazole (15 min)	15/162	168/175
0.10 μg/ml nocodazole (15 min)	7/182	247/293
0.10 μg/ml nocodazole (45 min)	2/164	184/285
4°C (120 min)	2/145	169/252
10°C (120 min)	27/176	241/232

* Numerator is the total number of neurites in surveyed regions on a single dish after treatment; denominator is the number of neurites in same regions before treatment.

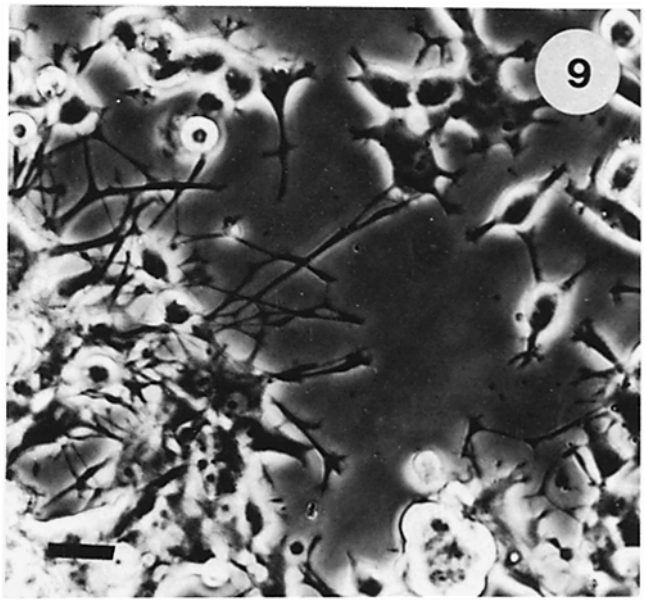

FIGURE 9 NC-culture cells before MT depolymerization. Cells were treated with NGF for 6 d, triturated, replated in medium containing NGF and 0.1 μM 2-chloroadenosine, and allowed to regenerate neurites for 24 h. Bar, 25 μm. × 400.

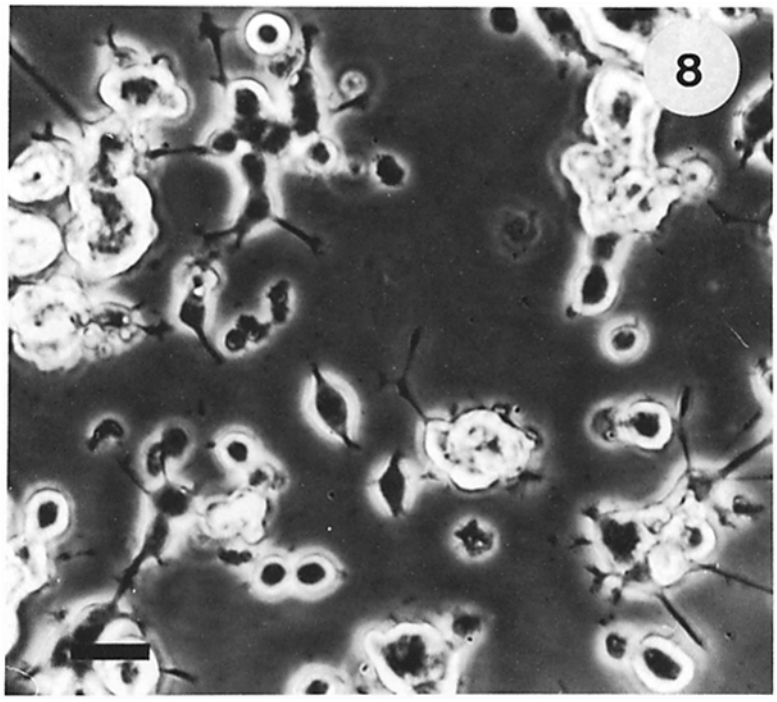

FIGURE 8 N-culture cells before MT depolymerization. Cells were treated with NGF for 6 d, triturated, replated in medium containing NGF, and allowed to regenerate neurites for 24 h. Bar, 25 μm. × 400.

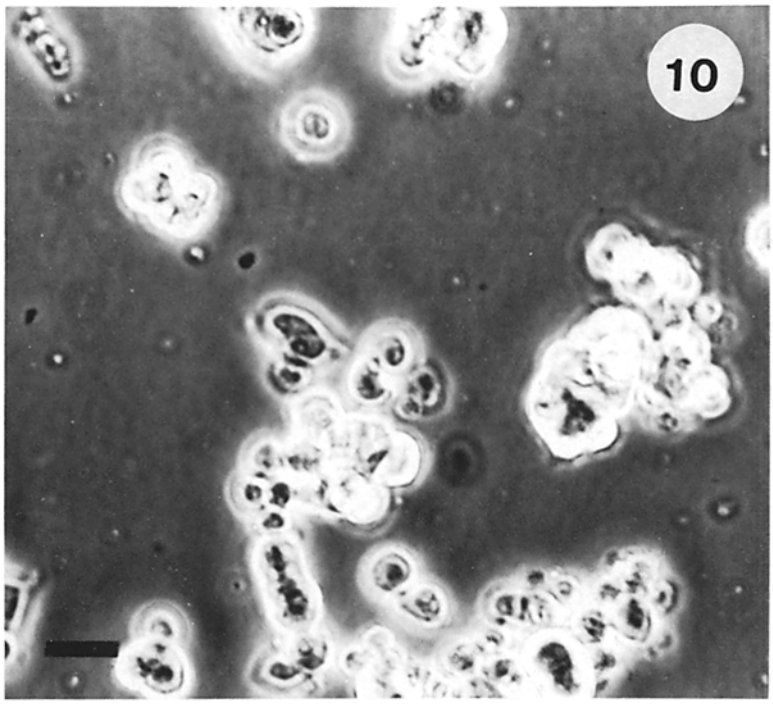

FIGURE 10 Retraction of N-culture neurites treated with nocodazole. Cells in the presence of NGF treated with 0.05 μg/ml nocodazole for 15 min. This photograph was taken of the same circled region as Fig. 8; see text for explanation. Bar, 25 μm. × 400.

proved to be less stable to MT depolymerization than NC-culture neurites regenerated for only 1 d. In two experiments, after 15 min in the presence of 0.1 μg/ml of nocodazole, 107 of 598 N-culture neurites remained. The matched NC-cultures retained 641 of 694 neurites.

The stability of neurites stimulated by cAMP alone (C-cultures) was also assessed. We found that use of polylysine coated, Primaria dishes permitted neurite outgrowth in cAMP of sufficient length to assay by phase microscopy. It was for this reason that such dishes were used in all of the experiments on stability of neurites to MT-depolymerization treatments. Fig. 16 shows a culture of PC12 stimulated by dbcAMP for 48 h. Neurite retraction in the presence of 0.1 μg/ml nocodazole after 15 min is shown in Fig. 17. In one such instance, 75 of 320 such neurites remained after this treatment. This indicates neurite stability similar to that for N-culture neurites allowed to regenerate for 3 d but clearly less than that for NC-cultures. A similar "intermediate" stability was seen for C-culture neurites exposed to low temperature. After 2 h at 4°C, C-cultures had largely retracted their neurites; 37/228 remained in a typical experiment. However, little or no retraction occurred in the same time period at 10°C; 260/280 neurites remained.

DISCUSSION

Published reports disagree as to whether or not NGF causes a significant elevation of cAMP levels in PC12 cells (18, 35). There is also disagreement about the extent to which dbcAMP mimics the effect of NGF (16, 18, 33–36). Although our results reveal certain similarities in the effects of dbcAMP and NGF on PC12 cell morphology (e.g., rapid loss of microvilli and transient membrane ruffling), we observed two synergistic effects of cAMP and NGF that provide strong evidence for divergent pathways of response to dbcAMP and NGF. The first is the dramatically enhanced neurite formation upon addition of NGF to cells already in the presence of sufficient dbcAMP to give a saturating response (Fig. 2). The second is the ability of added dbcAMP to significantly increase the stability of neurite MTs in cells that had been grown in NGF for 6 or 16 d. These synergistic effects cannot be due to insufficient permeability of cells to dbcAMP, or to some artifact arising from the use of a cAMP analogue, since a similar synergism of action is seen between NGF and cholera toxin or 2-chloroadenosine. These observations indicate that at least some of the aspects of PC12 responses to NGF and cAMP are independent. Acheson et al. (1) reached a similar conclusion in their study of enzyme induction in adrenal chromaffin cells mediated by NGF and cAMP.

While this manuscript was in preparation, Connolly et al. (10) compared the effects of dbcAMP and NGF on PC12 cells using scanning electron microscopy. Their results differ from ours in that they report that cAMP caused neither ruffling nor loss of microvilli. The membrane ruffling we observed after 2 min was transient and of limited extent. Thus, it may not represent a significant difference in the two studies. However, our observations on the loss of microvilli were consistent, and the loss was apparent throughout the first hour (Fig. 5*a*). We are unable to explain these differing observations since the two experimental protocols seem very similar.

The results reported here confirm the rapidity of NGF/cAMP synergism. As shown in Fig. 2, PC12 cells previously incubated with dbcAMP are stimulated by NGF to produce neurites within 5 min. This result puts useful limits on the types of mechanisms that need to be considered for some of the actions of NGF (i.e., the time scale over which causative biochemical changes need be sought). The improbability of new transcription followed by translation within this 5-min period supports the conclusion of Gunning et al. (16) that RNA synthesis is not required for NGF/cAMP synergistic response. The effect of NGF on PC12 cells may be mediated by both transcription dependent- and independent-events. Elevated cAMP levels may mimic or bypass the RNA synthesis-dependent event.

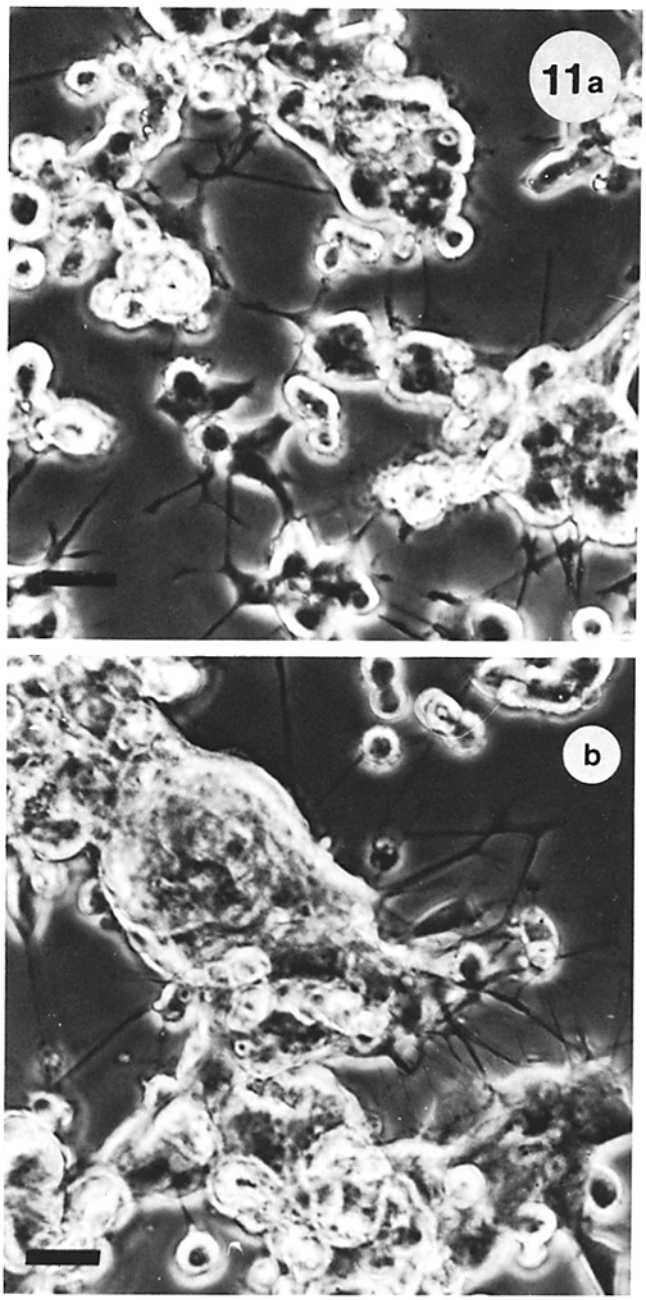

FIGURE 11 NC-culture neurites remain extended in nocodazole. PC12 cells in the presence of NGF and 2-chloroadenosine were treated with 0.1 μg/ml nocodazole for (*a*) 15 min or (*b*) 45 min. These cells were in the same circled region as those in Fig. 9. Bar, 25 μm. × 400.

Gunning et al. (16) speculated that the NGF/cAMP response of PC12 involves reorganization of the cytoskeleton. We adopted the well-established retraction of neurites in response to MT depolymerization (11, 37) as a measure of MT stability in the PC12 neurite. When possible, the effect of treatment on MTs was confirmed by electron microscopy. Low temperatures (23) and the synthetic MT drug nocodazole (13) were used as MT-depolymerization agents. Neurite stability in N- and NC-cultures was assessed for regenerated rather than newly initiated neurites (6). Use of regenerated neurites limited the number of differences between N- and NC-populations. Preliminary experiments comparing newly initiated neurites gave results similar to those reported here

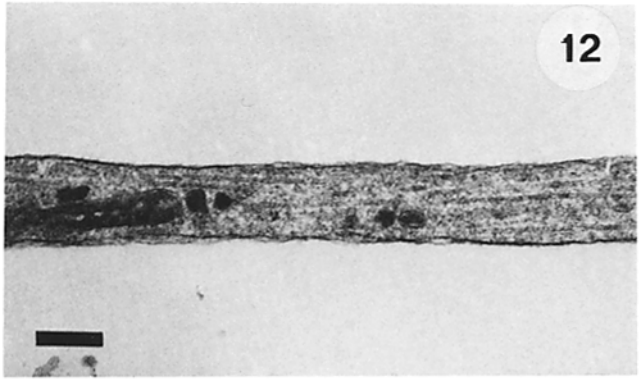

FIGURE 12 Neurites of NC-culture stable to nocodazole contain a normal complement of MTs. An electron micrograph of a neurite regenerated in the presence of NGF and 2-chloroadenosine, fixed after 45 min in 0.1 μg/ml nocodazole. Bar, 0.4 μm. × 23,000.

but were difficult to interpret due to unavoidable, multiple differences between cultures, such as different ages of neurites, duration of NGF stimulation, density of cells, etc. As shown in Table I, NC-culture neurites were significantly more stable than N-culture neurites to both cold- and poison-induced MT depolymerization. We interpret these results and our ultrastructural observations to mean that the MTs within NC-cells are more stable than MTs in N-cells. The low concentrations of nocodazole in these experiments had previously been shown to differentially depolymerize the nonkinetochore subpopulation of mitotic microtubules (19). On that basis, the difference in MT stability between N- and NC-cultures seems similar to the well-established difference between kinetochore and nonkinetochore subpopulations of mitotic MTs to a variety of depolymerization stimuli (5, 32). Stabilization of MTs is one of the well-described effects of dbcAMP (23, 30, 31). Indeed, neurites grown out in elevated concentrations of cAMP alone appeared to be intermediate between N- and NC-culture neurites in stability to MT depolymerization–induced retraction. It is interesting that PC12 treated with dbcAMP only or with chloroadenosine and grown on polylysine coated, Primaria plates formed neurites as long as 4 cell diameters within 48 h. On other substrata, PC12 produces only very short neurites in response to elevated concentrations of cAMP. Both Primaria plasticware and polylysine treatment increase cell adhesion, suggesting that lack of adhesiveness is a major factor in limiting neurite outgrowth by dbcAMP alone.

The results here suggest that the equilibrium or steady state of MT assembly in NC-culture neurites is shifted further toward assembly than in N- or C-cultures. That is, the critical concentration (22) for assembly of tubulin is lower in NC-cells than in either N- or C-cells. Although MT assembly is generally regarded as being near equilibrium or steady state in interphase cells, this conclusion is somewhat tentative in that our assay is essentially a kinetic measurement. In any case, in view of the important role MTs play in the outgrowth of the axon (11, 24, 37), it seems likely that the increase in MT stability in NC-cultures plays an important role in the greater rate and extent of neurite growth under this culture condition. Our interpretation is that neurite initiation is limited or delayed in N-cultures because the critical concentration of tubulin is too high to assemble appropriately to allow neurite growth. The work of Greene and colleagues on PC12 cells stimulated only by NGF is consistent with this sugges-

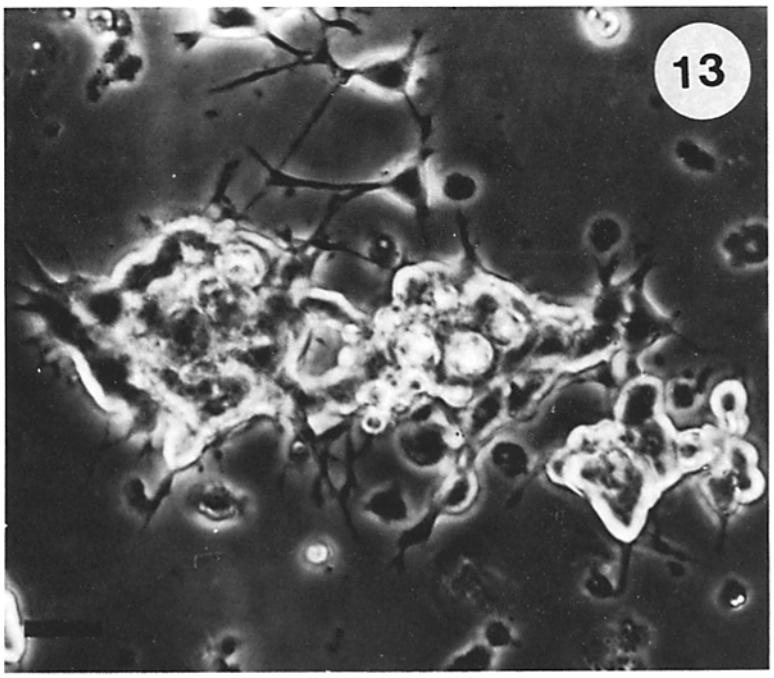

FIGURE 13 NC-culture neurites remain extended at low temperature. Cells in the presence of NGF and 2-chloroadenosine after 2 h at 4°. Bar, 25 μm. × 400.

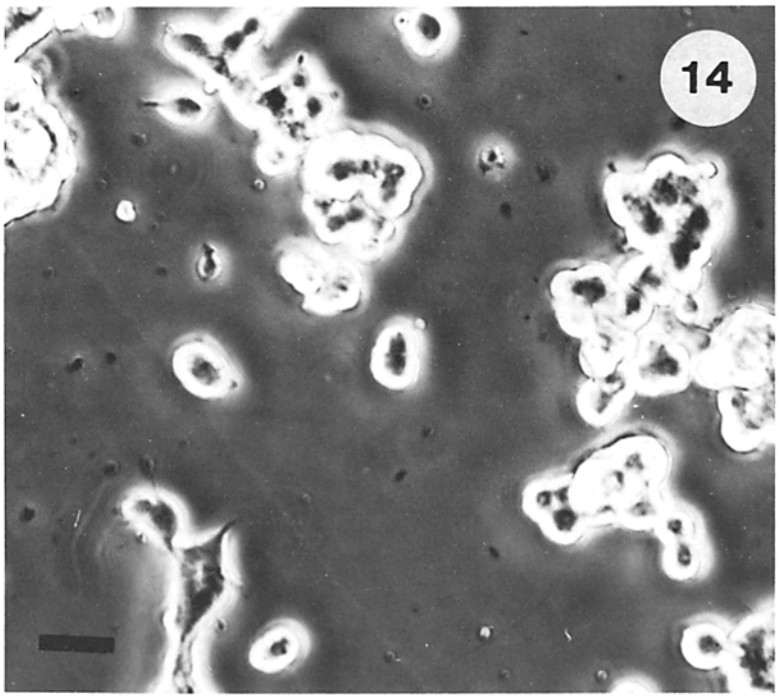

FIGURE 14 N-culture neurites retract at 10°C. Cells were cultured in the presence of NGF after 2 h at 10°C. Bar, 25 μm. × 400.

tion. They showed that an increase in MT stability to colchicine treatment accompanies the outgrowth of neurites (2). They also found (15) that NGF induces the synthesis of a microtubule-associated protein, some of which lower the critical concentration of tubulin. Olmsted et al. (29) have also found that microtubule-associated protein synthesis accompanies neurite outgrowth in neuroblastoma. One possibility is that the transcription- and translation-independent stabilization of NC MTs mimics the RNA-dependent synthesis of MT-stabilizing microtubule-associated proteins in N-cultures.

Whatever the mechanism that stabilizes MTs in NC-culture conditions, it is unclear how such MT stability is involved in the increase of the rate and extent of PC12-neurite growth. However, our results are consistent with the speculation of Bray (3) that external force could provide a link between MT stability and growth cone motility. The retraction of the neurite in response to MT depolymerization is consistent with MTs being under compression. The tension on the neurite (3, 24) may normally be supported by the compression of MTs. The disassembly of MTs disturbs this equilibrium and the neurite retracts in response to the tension. Hill and Kirschner have shown that the tubulin subunit-polymer equilibrium is shifted away from assembly by compression (22). An increase in microtubule stability, relative to a zero force situation, would be required to enable the MT to bear compression without depolymerization.

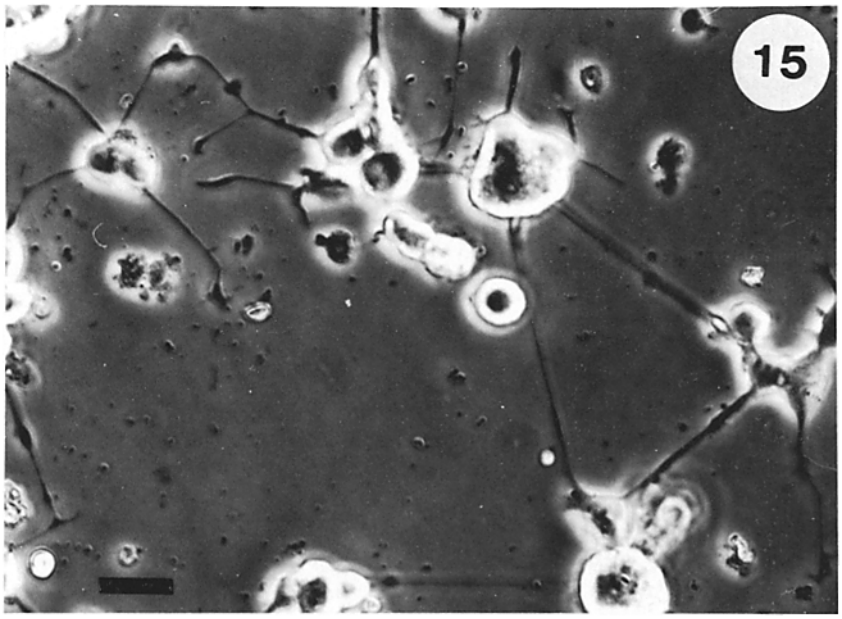

FIGURE 15 N-culture cells after 3 d of neurite regeneration. Cells were grown for 14 d in the presence of NGF, triturated, and replated in the presence of NGF for an additional 3 d. Note the greater extension of neurites here than after 24 h of neurite regeneration. Bar, 25 μm. × 400.

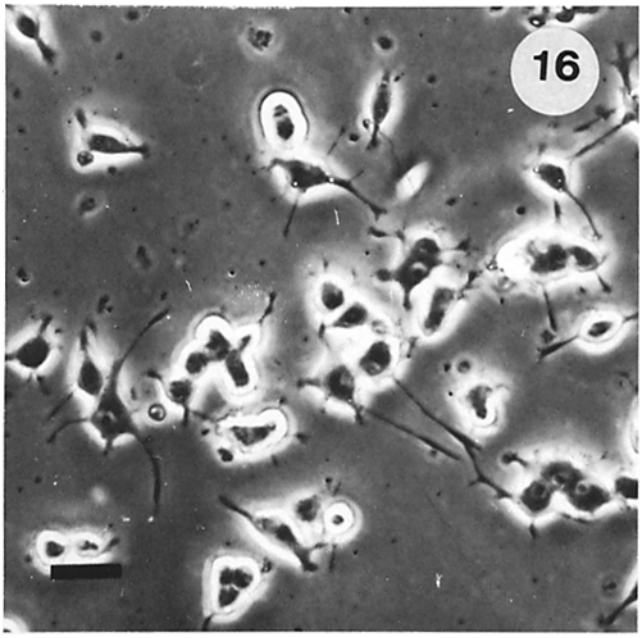

FIGURE 16 PC12 cells after 48 h in the presence of dbcAMP. These C-culture cells were never exposed to NGF. Bar, 25 μm. × 400.

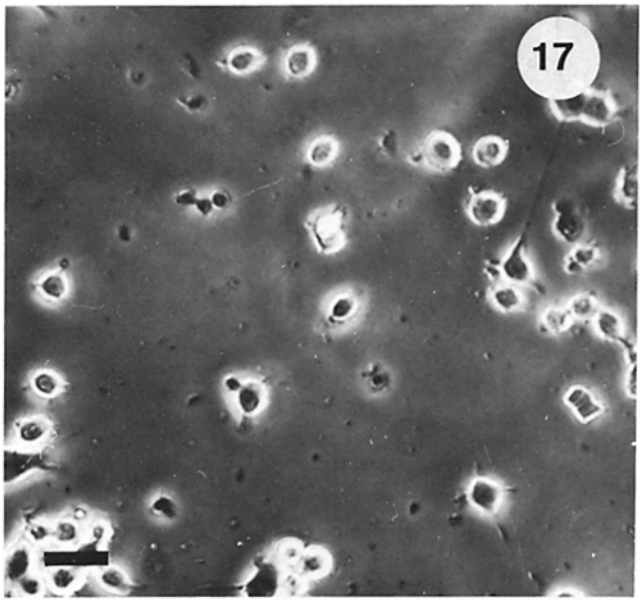

FIGURE 17 Neurites of C-culture cells substantially retract after 15 min with 0.1 μg/ml nocodazole. These cells were from the same circled region as in Fig. 16. Bar, 25 μm. × 400.

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